



(51) International Patent Classification ⁶ : A61K 9/127	A1	(11) International Publication Number: WO 96/08235 (43) International Publication Date: 21 March 1996 (21.03.96)
(21) International Application Number: PCT/US95/11609 (22) International Filing Date: 13 September 1995 (13.09.95) (30) Priority Data: 08/305,158 13 September 1994 (13.09.94) US (71) Applicant: DEPOTECH CORPORATION [US/US]; Suite 100, 11025 North Torrey Pines Road, La Jolla, CA 92037 (US). (72) Inventors: SANKARAM, Mantripragada, B.; 12726 Torrey Bluff Drive #53, San Diego, CA 92130 (US). KIM, Sinil; 548 Ford Avenue, Solana Beach, CA 92075 (US). (74) Agent: LEARN, June, M.; Fish & Richardson P.C., Suite 1400, 4225 Executive Square, La Jolla, CA 92037 (US).		(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG). Published <i>With international search report.</i>
(54) Title: PREPARATION OF MULTIVESICULAR LIPOSOMES FOR CONTROLLED RELEASE OF ACTIVE AGENTS (57) Abstract Disclosed are multivesicular liposomes (MVL's) containing biologically active substances, the multivesicular liposomes having defined size distribution, adjustable average size, adjustable internal chamber size and number, and a controlled and variable release rate of the biologically active substance. The process involves encapsulating at least one biologically active substance and optionally an osmotic spacer in a first aqueous component encapsulated within the liposomes. The rate of release of the active substance into the surrounding environment in which the liposomes are introduced can be decreased by increasing the osmolarity of the first aqueous component or increased by decreasing the osmolarity.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

PREPARATION OF
MULTIVESICULAR LIPOSOMES FOR CONTROLLED
RELEASE OF ACTIVE AGENTS

BACKGROUND OF THE INVENTION

5 **1. *Field of the Invention***

The present invention relates to a multivesicular liposome composition of liposomes encapsulating biologically active substances. More particularly, the present invention relates to methods to produce and use multivesicular liposomes with controllable release rate of the
10 encapsulated substance.

2. *Description of Related Art*

Optimal treatment with many drugs often requires that the drug level be maintained for a prolonged period of time. For example, optimal anti-cancer treatment with cell cycle-specific antimetabolites requires
15 maintenance of a cytotoxic drug level for a prolonged period of time. Cytarabine is a highly schedule-dependent anti-cancer drug. Because this drug kills cells only when they are synthesizing DNA, prolonged exposure at therapeutic concentration of the drug is required for optimal cell kill. The therapeutic effectiveness of such agents is often
20 further complicated by the fact that the half-life after an intravenous or subcutaneous dose may be as short as a few hours. To achieve optimal cancer cell kill with a cell cycle phase-specific drug like cytarabine, there are two major requirements: first, the cancer cell must be exposed to a high concentration of the drug without doing
25 irreversible significant harm to the host; and second, the tumor must be exposed to the drug for a prolonged period of time to maximize the number of cancer cells which are in the susceptible DNA synthesis cycle of cell proliferation.

An example of another class of drugs that are schedule-dependent is the aminoglycoside class of antibiotics. For instance, amikacin is an aminoglycoside antibiotic which has clinically significant activity against strains of both gram negative and gram positive bacteria. Under
5 existing therapeutic procedures, the drug is normally administered by intravenous or intramuscular routes on a once or twice a day schedule. The most commonly used clinical dose is 15 mg/Kg/day which is equivalent to a maximum recommended daily dose of 1 g per day. However, this approach results in systemic exposure to the patients,
10 and depending on the drug, increases risk of toxic side effects. Consequently, a local depot slow-release preparation for treatment of infections such as those confined to a local region of soft tissue or bone would be advantageous in increasing local tissue levels of the drug, compared with therapeutic systemic doses, while reducing or avoiding
15 the systemic toxicity of the free drug.

Especially useful would be controlled release preparations that can be used to deliver therapeutic levels of drugs over periods ranging from hours, days, or even weeks.

One approach which has been used to provide controlled release
20 compositions for drug delivery is liposome encapsulation. Among the main types of liposomes, multivesicular liposomes (Kim, *et al.*, *Biochim. Biophys. Acta*; 728:339-348, 1983), are uniquely different from unilamellar liposomes (Huang, *Biochemistry*, 8:334-352, 1969; Kim, *et al.*, *Biochim. Biophys. Acta*; 646:1-10, 1981), multilamellar liposomes
25 (Bangham, *et al.*, *J. Mol. Bio.*, 13:238-252, 1965), and stable plurilamellar liposomes (U.S. Patent No. 4,522,803). In contrast to unilamellar liposomes, multivesicular liposomes contain multiple aqueous chambers. In contrast to multilamellar liposomes, the multiple aqueous chambers of multivesicular liposomes are non-concentric.
30 The prior art describes a number of techniques for producing various

types of unilamellar and multilamellar liposomes; for example, U.S. Patent No. 4,522,803 to Lenk; 4,310,506 to Baldeschwieler; 4,235,871 to Papahadjopoulos; 4,224,179 to Schneider; 4,078,052 to Papahadjopoulos; 4,394,372 to Taylor; 4,308,166 to Marchetti; 5 4,485,054 to Mezei; and 4,508,703 to Redziniak; Szoka, *et al.*, 1980, *Ann. Rev. Biophys. Bioeng.*, 9:465-508; *Liposomes*, Marc J. Ostro, Ed., Marcel-Dekker, Inc., New York, 1983, Chapter 1; Poznansky and Juliano, *Pharmacol. Rev.*, 36:277-236, 1984. The prior art also describes methods for producing multivesicular liposomes (Kim, *et al.*, 10 *Biochim. Biophys. Acta*, 728:339-348, 1983).

In the method of Kim, *et al.*, (*Biochim. Biophys. Acta*, 728:339-348, 1983) the encapsulation efficiency of some small molecules such as cytosine arabinoside, also known as cytarabine or Ara-C, was relatively low, and the release rate of encapsulated molecules in biological fluids 15 was high. Subsequently, a composition was developed which used hydrochloride to slow the release rate of encapsulated molecules in biological fluids (U.S. Patent continuation-in-part application of application Serial No. 08/020,483, filed February 21, 1993, by Kim).

Further research has shown that the release rate of substances from multivesicular liposomes in human plasma can be controlled by means 20 of varying the nature of the acid solution in which the substance is dissolved prior to forming the multivesicular liposome (U.S. Patent application Serial No. 08/153,657, filed November 16, 1993, Sankaram and Kim). In these studies, certain mineral acids such as hydrochloric, 25 nitric and perchloric acids produced the slowest release rates, whereas other acids, such as acetic, trifluoroacetic, and trichloroacetic resulted in intermediate release rates. The fastest release rates were obtained using di- and tri-protic acids such as sulfuric and phosphoric acids. The composition of liposomes produced by this method has the 30 disadvantage that it requires the use of acid to produce a multivesicular

liposome with desirable drug release kinetics. Furthermore, some of the acids which might provide desirable drug release rates from multivesicular liposomes may present pharmaceutical process problems, such as corrosion of metallic containers and parts used in the manufacture. Also, potential problems with the stability of acid-labile substances could be avoided if multivesicular liposomes which are practical in an *in vivo* environment could be produced without the use of acid.

Studies have shown that the rate of release of the encapsulated biological substance from liposomes into an aqueous environment can be modulated by creating a membrane potential by introducing protonophores or ionophores into liposomes (U.S. Patent No. 5,077,056). In addition, a method for controlling the release rate of drugs from vesicle compositions is disclosed in European Patent Application EP 0 126 580. In this latter method, a composition was provided comprising a solution of a therapeutic agent encapsulated in vesicles which were suspended in a solution containing sufficient solute to provide a certain osmolarity. This osmolarity was at least substantially isotonic with respect to the osmolarity of the solution within the vesicles which, in turn, had a greater osmolarity than physiological saline. The osmolarity of the solution within the vesicles was held constant, while the osmolarity of the solution in which the vesicles are suspended was varied. Under these conditions, the release rate decreased as the suspending medium approached an isotonic, or even hypertonic relationship with respect to the solution within the vesicles. However, a drawback of this method is that the osmolarity of the medium into which the therapeutic agent is released must be varied in order to control the rate of release. As a consequence, these compositions are severely limited in terms of their practical use, for example, in pharmaceutical applications, since the osmolarity of the biological fluid at the site of administration of the drug

delivery system is substantially fixed and cannot be varied. For example, biological fluids such as plasma, are close to isotonic with respect to normal saline (0.9 wt% NaCl in water), which has an osmolality of 310 mOsm. Therefore, the osmolality of the suspending medium should ideally be close to 310 mOsm.

In view of the perceived limitations with existing liposome compositions and production methodology, techniques which do not rely upon the use of acids for production of stable multivesicular liposomes are needed. The present invention addresses this need.

10 SUMMARY OF THE INVENTION

The present invention provides multivesicular liposomes (MVL's) with controlled release of a biologically active substance. The rate of release of the biologically active substance into the physiological environment, such as human tissue *in vivo*, is controlled by varying the osmolality of the first aqueous component which is encapsulated within the liposomes. In so doing, the MVL's of the invention achieve greatly improved results by providing a sustained therapeutic release of drug when introduced to an *in vivo* site. Unexpectedly, as the osmolality of the first aqueous component which is encapsulated within the liposomes is increased, the rate of release decreases.

Osmolality of the first aqueous component can be increased either by increasing the concentration of the biologically active substance or by addition of an osmotic spacer.

The present invention also provides non-acidic methods for making multivesicular liposomes with controlled release characteristics which provide for prolonged *in vivo* therapeutic use. The multivesicular liposomes of the invention have high encapsulation efficiency, controlled release rate of the encapsulated substance, well defined,

reproducible size distribution, spherical shape, easily adjustable average size and adjustable internal chamber size and number.

5 The process of the invention for producing multivesicular lipid vesicles or multivesicular liposomes comprises (a) dissolving in one or more organic solvents a lipid component containing at least one amphipathic lipid and one neutral lipid, (b) mixing the lipid component with an immiscible first aqueous component containing one or more biologically active substances to be encapsulated, (c) forming a water-in-oil emulsion from the two immiscible components, (d) transferring and
10 immersing the water-in-oil emulsion into a second immiscible aqueous component forming solvent spherules, and (e) removing the organic solvents, such as by evaporation, from the solvent spherules. The amphipathic lipid(s) can be selected from amphipathic lipids with a net negative charge; sterols; and zwitterionic lipids. The release rate of
15 encapsulated molecules into biological fluids and *in vivo* can be decreased either by increasing the concentration of the biologically active substance, by using an osmotic spacer or by combined use of increased substance and osmotic spacer.

DESCRIPTION OF PREFERRED EMBODIMENTS

20 The present invention is directed to multivesicular liposome (MVL) compositions which allow controlled release of therapeutically active substance in biological fluids. In these compositions the osmolarity of the encapsulated aqueous phase is manipulated in a novel manner to achieve sustained release of the encapsulated therapeutically active
25 substance in the absence of hydrochlorides or acids. By manipulating the process of the invention one of skill in the art can selectively produce MVL's with a broad range of release rates for the substance.

The term "multivesicular liposomes" means man-made, microscopic lipid vesicles enclosing multiple non-concentric aqueous chambers. In contrast, other previously described liposomes such as unilamellar liposomes have a single aqueous chamber and multilamellar and plurilamellar liposomes have multiple concentric "onion-skin"-like membranes in between which are spaces containing an aqueous component.

The term "solvent spherule" means a microscopic spheroid droplet of organic solvent, within which are multiple smaller droplets of the aqueous component containing the biologically active agent. The solvent spherules are suspended and totally immersed in a second aqueous component.

The term "neutral lipid" means oil or fats that have no vesicle-forming capability by themselves and lack a charged or hydrophilic "head" group. Examples of neutral lipids include, but are not limited to, glycerol esters, glycol esters, tocopherol esters, sterol esters which lack a charged or hydrophic "head-group", and alkanes and squalene.

The term "amphipathic lipids" means those molecules that have a hydrophilic "head" group and hydrophobic "tail" group and may have membrane-forming capability. As used herein, amphipathic lipids include those amphipathic lipids having a net negative charge, a net positive charge, zwitterionic lipids, and sterols.

The term "zwitterionic lipid" means an amphipathic lipid with a net charge of zero at its isoelectric point.

As used herein, the term "biologically active", when used to describe substances present in the chambers of the multivesicular liposome, includes substances which possess biological activity in the form as

presented in the vesicle as well as substances which become active after release from the vesicle chamber (i.e., possess "quiescent" biological activity), such as a pro-drug which is converted upon interaction with an enzyme into an active moiety with therapeutic activity.

In addition, biologically active substances which can be incorporated include substances which act indirectly. Alternatively, such substances may act as an osmotic spacer as described herein. For example, various excipients and stabilizers may be present. Such substances may act, for example, to increase the shelf life or bioavailability of a particular drug. Alternatively, many substances commonly classified as excipients may actually possess direct biological activity from very slight to quite significant. For example, the common excipient mannitol can also act biologically as a diuretic and even water may act biologically to affect dehydration. Such indirectly active substances include aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solutions are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic-aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose, and lactated Ringer's solution. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like (see, Remington's Pharmaceutical Sciences, 16th Ed., A. Oslo, ed., Mack, Easton, PA. 1980). Those of ordinary skill in the art can readily ascertain and envision various combinations of compounds which can be utilized in the vesicles of the invention without resorting to undue experimentation.

Osmolarity is defined as the sum of molar concentrations of solutes present in an aqueous solution. If the solute is present in a dissociated, ionized or aggregated form, osmolarity is defined as the sum of the molar concentrations of the dissociated, ionized or aggregated forms. The solutes include, but are not limited to, biologically active agents and osmotic spacers.

The term "osmotic spacer" means any biologically compatible solute molecule, in an aqueous solution, that is not the biologically active substance. Both electrolytes and non-electrolytes function as osmotic spacers. In determining whether any particular molecule will function as an osmotic spacer or in determining the concentration of osmotic spacer encapsulated within a multivesicular liposome, consideration must be given to whether, under conditions within the multivesicular liposome (for example, pH), the molecule is wholly or partially ionized and whether such ions will permeate the lipid membrane (The Bimolecular Lipid Bilayer Membrane, Mahendra K. Jain, van Nostrand Reinhold Co., 1972, 470 pp.). One skilled in the art will appreciate that for use in the present invention, the osmotic spacer must be selected so as to avoid substances that would prove toxic or otherwise harmful to a subject undergoing therapy by use of the MVL of this invention. Those of skill in the art can readily evaluate the suitability of a given osmotic spacer for use in the present invention without resort to undue experimentation.

The term "first aqueous component" means the aqueous phase containing the biologically active substance, optionally in the presence of an osmotic spacer, used in the process to prepare multivesicular liposomes.

The term "osmolarity of the first aqueous component" means the osmolarity of the aqueous component containing the biologically active substance used in the process to prepare multivesicular liposomes.

5 The term "physiologically relevant aqueous environment" means biological fluids such as plasma, serum, cerebrospinal, intramuscular, subcutaneous, peritoneal, intraocular, or, synovial fluids, and storage media such as 0.9 wt% saline or buffered saline that are nearly isotonic with respect to the biological fluids.

10 The prior art methods of controlling the rate of release of the encapsulated biological substance into the aqueous environment rely on 1.) the nature of the acid solution within the liposomes, or 2.) increasing the osmolarity of the external aqueous environment into which the liposomes are introduced with respect to the osmolarity of the aqueous component within the liposomes. The logical corollary of
15 the latter method is that the rate of release of the encapsulated substance should increase as the osmolarity of the first aqueous component is increased while keeping constant the osmolarity of the aqueous medium into which the liposomes are to be introduced. However, the findings of the present invention surprisingly are the
20 opposite of what is expected based on the prior art teachings, namely, that the rate of release of the encapsulated substance actually decreases as the osmolarity of the internal aqueous component within the MVL's increases even though the osmolarity of the external aqueous medium remains constant. Accordingly, the method of this
25 invention uses osmolarity of the first aqueous component, which is encapsulated within the multivesicular liposomes, to control release where the osmotic strength of the external medium is near physiological.

In addition, when MVL's are produced which include an osmotic spacer, at a given concentration of the biologically active substance, increasing the concentration of osmotic spacer will unexpectedly decrease the release rate of the substance. The decrease in release rate with increase in osmolarity of the first aqueous component enhances the utility of the multivesicular liposomes for any type of therapeutic treatment that benefits from prolonged release of the therapeutic agent.

To decrease the release rate of encapsulated therapeutic or other active agents from the multivesicular liposomes of the invention, the osmolarity of the first aqueous component within the MVL's is increased relative to that of the physiologically relevant aqueous environment in which the MVL's are stored (such as 0.9 wt% saline) or into which the MVL's are introduced (both *in vitro* and *in vivo*) either by increasing the concentration of the active substance or by use of an osmotic spacer. Conversely, the release rate can be increased either by decreasing the concentration of the active substance, or by decreasing the concentration of, or eliminating the use of, an osmotic spacer. The osmolarity of the first aqueous component can also be hypertonic with respect to the physiologically relevant aqueous environment to provide an optimum decrease in the rate of release of the biologically active substance from the liposomes accompanied by a greater yield for the process of encapsulation than under isotonic conditions. Therefore, it is contemplated within the scope of this invention that the first aqueous component can be hypotonic, isotonic or hypertonic with respect to the storage medium or the aqueous environment into which the biologically active agent is to be released. Consequently, one of skill in the art can routinely produce an MVL with a pre-selected rate of release of the biologically active substance which is optimal for a given therapy.

Since the osmolality of normal saline is similar to that of human plasma and other *in vivo* environments, such as cerebrospinal fluid, synovial fluid, as well as subcutaneous and intramuscular spaces, saline can be used as a predictive model of MVL drug release in such environments. The preferred use of the MVL's of the invention is for *in vivo* injection or implantation into tissue or body cavities (for instance, as drug depots) and they are preferably stored in a medium such as normal saline, phosphate-buffered saline, or other osmotically similar medium.

10 Briefly, in the method embraced by the present invention MVL's are produced by first forming a water-in-oil emulsion made by dissolving the amphipathic lipid(s) and the neutral lipid in a volatile organic solvent for the lipid component, adding to the lipid component an immiscible first aqueous component containing a biologically active substance to
15 be encapsulated and then emulsifying the mixture, for example, through turbulence created by mixing, shaking, sonication, by ultrasonic energy, nozzle atomization, or by combinations thereof. The entire emulsion is then immersed in a second aqueous component and then agitated mechanically, as above, to form solvent spherules
20 suspended in the second aqueous component. The resulting solvent spherules are made up of multiple aqueous droplets in which the biologically active substance is contained (a water-in-oil-in-water double emulsion).

25 The volatile organic solvent is removed from the spherules, for example, by passing a stream of gas over or through the suspension to evaporate the solvent. When the solvent is completely evaporated, multivesicular liposomes are formed. Representative gases satisfactory for use in evaporating the solvent include nitrogen, helium, argon, oxygen, hydrogen and carbon dioxide. Alternatively the organic
30 solvent can be removed by solvent removal systems commonly

employed such as sparging with the above gases, evaporation under reduced pressure, and spray drying.

Osmotic spacers, which include, but are not limited to, glucose, sucrose, trehalose, succinate, cyclodextrin, arginine, galactose, mannose, maltose, mannitol, glycine, lysine, citrate, sorbitol, dextran, and combinations thereof, may be used to form multivesicular liposomes and to control the release rate of the encapsulated substance from multivesicular liposomes. The concentration of the encapsulated biologically active substance can vary from about a few picomoles to about several hundred millimoles. The concentration of biologically active substance will vary depending upon such characteristics as the disease to be treated, age and condition of the patient, as well as the particular properties of the substance. In the case where the substance is normally associated with side effects such as toxicity it might be desirable to produce an MVL with a lower concentration of substance and utilize a higher concentration of osmotic spacer. The interrelationship of these various parameters can be easily evaluated by one of skill in the art in selecting and producing a given MVL composition without resort to undue experimentation.

Many different types of volatile hydrophobic solvents such as ethers, hydrocarbons, halogenated hydrocarbons, supercritical fluids including but not limited to CO_2 , NH_3 , and freons may be used as the lipid-phase solvent. For example, diethyl ether, isopropyl and other ethers, dichloromethane, chloroform, tetrahydrofuran, halogenated ethers, esters and combinations thereof are satisfactory.

Various types of lipids can be used to make the multivesicular liposomes as long as at least one neutral lipid and at least one amphipathic lipid are included. The amphipathic lipid(s) can be selected from the group consisting of (1) amphipathic lipids with a net

negative charge; (2) sterols; and (3) zwitterionic lipids. For instance, the lipid component can comprise a neutral lipid and an amphipathic lipid with a net negative charge. In an alternative embodiment, the lipid component further comprises a sterol. And in yet another alternative
5 embodiment, the lipid further comprises both a sterol and a zwitterionic lipid.

Examples of zwitterionic lipids are phosphatidylcholines, phosphatidylethanolamines, sphingomyelins, and combinations thereof. Examples of neutral lipids are triglycerides such as triolein, tricaprylin,
10 vegetable oils such as soybean oil, lard, beef fat, tocopherol, squalene and combinations thereof. Examples of amphipathic lipids with net negative charge are cardiolipins, phosphatidylserines, phosphatidylglycerols, phosphatidylinositols, and phosphatidic acids. Examples
of amphipathic lipids with a net positive charge are diacyl trimethyl-
15 ammonium propanes, diacyl dimethylammonium propanes and stearylamine.

Various biologically active substances can be incorporated by encapsulation within the multivesicular liposomes. These substances include drugs, as well as other kinds of pharmaceutical materials such
20 as DNA, RNA, proteins of various types, protein hormones such as insulin, growth factors, cytokines, monokines, lymphokines, and proteins and carbohydrates that serve as immunogens for vaccination.

Biologically active substances effective for cosmetic uses can also be incorporated by encapsulation into multivesicular liposomes. These
25 include moisturizers, vitamins, enzymes, and perfumes. In addition, herbicides, pesticides, fungicides etc. are examples of biologically active substances with agricultural uses that can be encapsulated into multivesicular liposomes.

Table 1 includes representative classes of biologically active substances that can be encapsulated in the multivesicular liposomes of this invention.

TABLE 1

5	Antianginas	Antiarrhythmics	Antiasthmas
	Antibiotics	Antidiabetics	Antifungals
	Antihistamines	Antihypertensives	Antiparasitics
	Antineoplastics	Antivirals	Cardiac Glycosides
10	Herbicides	Hormones	Immunomodulators
	Monoclonal Antibodies	Neurotransmitters	Nucleic Acids and Analogs
	Pesticides	Proteins and Glycoproteins	Radio Contrast Agents
	Radionucleids	Sedatives and Analgesics	Steroids
15	Tranquilizers	Vaccines	Vasopressors
	Perfumes	Cosmetics	Moisturizers
			Fungicides

The dosage range appropriate for *in vivo* use in humans of the biologically active substance in multivesicular liposomes of this invention includes the range of 0.001-6,000 mg/m² of body surface area. While doses outside the foregoing dose range may be given, this range encompasses the breadth of use for practically all the biologically active substances. However, for a particular therapeutic agent the preferred concentration can be easily ascertained as previously described.

The multivesicular liposomes may be administered by any desired route; for example, intratumoral, intra-articular (into joints), intramuscular, intrathecal, intraperitoneal, subcutaneous, intravenous, intralymphatic, oral and submucosal. The multivesicular liposomes
5 may be modified using methods well known in the art by attaching thereto, either directly, or indirectly, such as by means of a spacer molecule or peptide, target-specific ligands, such as antibodies and other receptor specific protein ligands, in order to impart organ or cell target specificity (Malone, *et al.*, Proc. Nat'l. Acad. Sci, U.S.A., 86:6077,
10 1989; Gregoriadis, Immunology Today, 11(3):89, 1990; both incorporated by reference).

The following examples illustrate the manner in which the invention can be practiced. It is understood, however, that the examples are for the purpose of illustration and the invention is not to be regarded as limited
15 to any of the specific materials or conditions therein.

EXAMPLE 1

A. Preparation of Multivesicular Liposomes.

Step 1) In a clean glass cylinder (2.5 cm inner diameter X 10.0 cm height), were placed 5 mL of a solution containing in chloroform 46.5
20 μ moles of dioleoyl phosphatidylcholine (Avanti Polar Lipids, Birmingham, AL), 10.5 μ moles of dipalmitoyl phosphatidylglycerol (Avanti Polar Lipids, Birmingham, AL), 75 μ moles of cholesterol (Sigma Chemical Co., St. Louis, MO), 9.0 μ moles of triolein (Avanti Polar Lipids, Birmingham, AL). This solution is referred to as the lipid
25 component.

Step 2) Five mL of either first aqueous component, cytarabine (Upjohn, Kalamazoo, MI), or amikacin sulfate (Bristol Myers Squibb,

Syracuse, NY) dissolved in water, were added into the above glass cylinder containing the lipid component. For cytarabine, the concentrations used were 41 mM, 82 mM, 164 mM, 246 mM, 369 mM, and 410 mM. For amikacin sulfate, the concentrations used were 13 mM, 26 mM, 52 mM, and 88 mM. Amikacin sulfate contains 1.8 sulfate ions per amikacin molecule. Thus, the concentration is multiplied by 2.8 to obtain the osmolarity.

Step 3) For making the water-in-oil emulsion, the mixture of step 2 was stirred with an homogenizer (AutoHomoMixer, Model M, Tokushu Kika, Osaka, Japan) for 8 minutes at a speed of 9000 rpm.

Step 4) For making the chloroform spherules suspended in water, 20 mL of a solution containing 4 percent dextrose and 40 mM lysine was layered on top of the water-in-oil emulsion, and then mixed for 60 seconds at a speed of 4000 rpm to form the chloroform spherules.

Step 5) To obtain the multivesicular liposomes, the chloroform spherule suspension in the glass cylinder was poured into a 1000 mL Erlenmeyer flask containing 30 mL of water, glucose (3.5 g/100 mL), and free-base lysine (40 mM). A stream of nitrogen gas at 7 L/minute was passed over the suspension in the flask to slowly evaporate chloroform over 20 minutes at 37°C. Sixty mL of normal saline (0.9% sodium chloride) was added to the flask. The liposomes were then isolated by centrifugation at 600 X g for 10 minutes, the supernatant was decanted, and the pellet was resuspended in 50 mL of normal saline. The liposomes were isolated by centrifugation at 600 X g for 10 minutes. The supernatant was decanted, and the pellet was resuspended in saline.

B. Determination of Mean Diameter of Multivesicular Liposomes

The mean diameter of multivesicular liposomes prepared as described above was determined by the method of laser light diffraction using the
5 model LA-500 Particle Size Analyzer from Horiba, Inc. (Irvine, CA). The mean diameter of the resulting liposomes was in the range from 5 to 20 μm .

C. Plasma Release Assay for Cytarabine

This example demonstrates that the rate of release of cytarabine and
10 amikacin in human plasma at 37 °C decreases with increasing concentration of the drug in the first aqueous component.

Five hundred μL of the multivesicular liposome suspension containing cytarabine was added to 1.2 mL of type O, screened human plasma. The mixture was incubated at 37 °C. At desired time intervals, an
15 aliquot was taken, the multivesicular liposomes were isolated as a pellet by centrifugation at 600 x g, the pellet was solubilized in isopropyl alcohol, and the amount of cytarabine in the pellet fraction was assayed by high pressure liquid chromatography (e.g. U.S. Pharmacopeia, XXII, p.376, 1990).

D. Plasma Release Assay for Amikacin

Five hundred μL of the multivesicular liposome suspension containing amikacin were added to 1.2 mL of type O, screened human plasma. The mixture was incubated at 37 °C and at desired time intervals an
25 aliquot was taken. The multivesicular liposomes were isolated as a pellet by centrifugation at 600 x g. The pellet was solubilized in isopropyl alcohol and the amount of amikacin in the pellet fraction was

assayed by a polystyrene bead based fluorescence immunoassay (Varma-Nelson, *et al.*, *Therapeutic Drug Monitoring*, 13:260-262, 1991). The percent retained amikacin was calculated from the amounts obtained by this procedure.

- 5 As can be seen in Table 2 encapsulation yield, expressed as a percentage of total drug used in the first aqueous component at different initial concentrations or osmolarities, had a marked influence on the rate of their release from the multivesicular liposomes incubated in human plasma, which is shown as half-life. The half-life of drug
- 10 release was calculated assuming a simple-exponential decay model. The data in Table 2 is the mean and standard deviation from three experiments. The half-life for release of cytarabine into human plasma remains essentially unchanged under hypertonic conditions relative to the isotonic condition. The data also show that the cytarabine and
- 15 amikacin half-life values increase with increasing concentration of the biologically active substance (shown as mOsm) encapsulated in the multivesicular liposomes during manufacture up to about 246 mOsm. Increasing the osmolarity of the first aqueous component, which is encapsulated within the multivesicular vesicles unexpectedly results in
- 20 producing liposomes with enhanced utility for slow release of drugs over long periods of time in human plasma.

TABLE 2

	Drug	Osmolarity (mOsm)	Mean Diameter (μ m)	Encapsul- ation Yield	Half-Life (Days)
	Cytarabine	41	9.1 ± 0.7	18 ± 3	1.0 ± 0.3
		82	10.9 ± 1.1	37 ± 2	1.8 ± 0.3
		164	13.3 ± 1.6	51 ± 4	4.6 ± 1.9
		246	14.7 ± 1.8	61 ± 5	14.1 ± 1.5
		369	16.1 ± 2.4	62 ± 3	12.1 ± 2.7
		410	13.4 ± 2.3	57 ± 2	13.0 ± 3.6
5	Amikacin sulfate	36	7.0 ± 1.2	35 ± 5	6.4 ± 2.7
		73	7.1 ± 1.2	42 ± 5	23.9 ± 1.4
		146	7.6 ± 1.1	50 ± 6	39.6 ± 2.9
		246	8.3 ± 1.7	66 ± 6	47.3 ± 4.0

EXAMPLE 2

This example demonstrates that the release rate of cytarabine in plasma decreases with increasing osmolarity of the first aqueous component at a fixed concentration of the biologically active substance. Multivesicular liposomes were prepared as described in Step 1 through Step 5 for Example 1, with the following modification for Step 2.

Step 2) Five mL of aqueous component, cytarabine (Upjohn, Kalamazoo, MI), dissolved at a concentration of 82 mM either in water, or in 3 wt% glucose, or in 5 wt% glucose, was added into the above glass cylinder containing lipid component. The calculated osmolarities of the resulting solutions are 82, 220 and 335 mOsm, respectively.

The percent of cytarabine retained in multivesicular liposomes after incubation at 37°C in human plasma for the different glucose concentrations was measured as a function of time of incubation. Table 3 shows the mean diameter, encapsulation yield of cytarabine and the half-life for release in plasma at 37°C for the compositions containing 0, 3 and 5 wt% glucose. The mean and standard deviation from three experiments is shown in each case. Incorporation of the osmotic spacer, glucose, at different concentrations while the biologically active substance is held at a fixed concentration had a marked influence on the rate of release from the multivesicular liposomes incubated in human plasma. As illustrated by the data in Table 3, the half-life for release of cytarabine from multivesicular liposomes into human plasma increases with increasing osmolarity of the first aqueous component. The encapsulation yield also increases with increasing osmolarity of the first aqueous component up to about 248 mOsm.

TABLE 3

	[Glucose] (wt%)	Osmolarity ^a (mOsm)	Mean Diameter (μm)	Encapsul- ation Yield	Half-Life (Days)
20	0	82	10.9 ± 1.1	37 ± 2	1.8 ± 0.3
	3	248	16.5 ± 1.6	55 ± 3	18.6 ± 5.7
	5	359	17.9 ± 1.1	57 ± 4	17.9 ± 4.3

^aOsmolarity includes both cytarabine and glucose.

EXAMPLE 3

25 This example demonstrates that multivesicular liposomes can be prepared when a normal saline solution is used for the second aqueous

component. Multivesicular liposomes were prepared as described below.

Step 1) In a clean glass cylinder (2.5 cm inner diameter X 10.0 cm height), 5 mL of a solution containing 46.5 μ moles of dioleoyl phosphatidylcholine (Avanti Polar Lipids, Birmingham, AL), 10.5 μ moles of dipalmitoyl phosphatidylglycerol (Avanti Polar Lipids, Birmingham, AL), 75 μ moles of cholesterol (Sigma Chemical Co., St. Louis, MO), 9.0 μ moles of triolein (Avanti Polar Lipids, Birmingham, AL) in chloroform were placed. This solution is referred to as the standard lipid component.

Step 2) Five mL of first aqueous component, cytarabine (Upjohn, Kalamazoo, MI) dissolved in water at a concentration of 246 mM, was added into the above glass cylinder containing the standard lipid component.

Step 3) For making the water-in-oil emulsion, a homogenizer (AutoHomoMixer, Model M, Tokushu Kika, Osaka, Japan) was used by mixing for 8 minutes at a speed of 9000 rpm.

Step 4) For making the chloroform spherules suspended in water, 20 mL of normal saline (0.9% sodium chloride) was layered on top of the water-in-oil emulsion, and then mixed for 60 seconds at a speed of 4000 rpm to form the chloroform spherules. This constitutes the water-in-oil-in-water double emulsion.

Step 5) The chloroform spherule suspension in the glass cylinder was poured into a 1000 mL Erlenmeyer flask containing 30 mL of normal saline (0.9 % sodium chloride). A stream of nitrogen gas at 7 L/minute was passed over the suspension in the flask to evaporate chloroform over 20 minutes at 37 °C. The liposomes were filtered

through a 50 μ m filter. The liposomes were isolated from the suspension by centrifugation at 600 X g for 10 minutes. The supernatant was decanted, and the pellet was resuspended in saline.

5 The half-life for release of cytarabine from multivesicular liposomes prepared using a 246 mOsm cytarabine solution in water, and normal saline for the second aqueous component, was 15.7 ± 4.8 days. This value is similar to the half-life for release from multivesicular liposomes prepared by using a 246 mOsm cytarabine solution in water, and a glucose-lysine solution for the second aqueous component (14.1 ± 1.5 days, see Table 2).

EXAMPLE 4

Pharmacokinetic studies in mice were performed to test the slow-release properties of the multivesicular liposomes of the invention *in vivo*.

15 Multivesicular liposomes were prepared as described in Example 1. Cytarabine solutions in water at concentrations of 82 mM or 246 mM were used as the first aqueous component. The multivesicular liposomes obtained were washed twice with normal saline, centrifuged at 600 x g for 10 minutes, and stored in normal saline at a
20 concentration of 10 mg cytarabine per mL of suspension.

Mice (CD1ICR, outbred, female) were injected intraperitoneally with multivesicular liposomes containing 2.55 ± 0.25 mg of cytarabine. Before injection, aliquots of the suspension of multivesicular liposomes containing the same amount of cytarabine as intended for injection
25 were taken as time zero samples.

At different time points up to 108 hours after injection, animals were sacrificed by cervical dislocation. Twenty μL of undiluted peritoneal fluid was recovered from the peritoneal cavity into a sample tube containing 200 μL of normal saline. The sample tube was centrifuged
5 in an Eppendorf Microfuge (Brinkman Instruments, Westbury, NY) at maximum speed for 1 minute. The supernatant and pellet were separated. After recovering 20 μL of peritoneal fluid as described above, the peritoneal cavity was washed out thoroughly with 2-3 ml of 0.9% NaCl solution. All samples were stored at -20°C before analysis.

10 The pellet and supernatant fractions, and the wash fraction were solubilized with isopropyl alcohol. The amount of cytarabine in the solubilized fractions was assayed by high pressure liquid chromatography (e.g. *U.S. Pharmacopeia*, XXII:376, 1990). The concentrations of cytarabine ($\mu\text{g}/\text{mL}$) in the pellet fraction are given in
15 Table 4. The total amounts of cytarabine (μg) in the intraperitoneal cavity, which are also given in Table 4, were obtained by adding the amounts in the pellet and supernatant fraction of the 20 μL undiluted peritoneal fluid sample, and the amount in the peritoneal wash fraction.

20 The pharmacokinetic data, i.e. the time-dependent decrease in cytarabine concentrations in the pellet and supernatant fractions and in the total amount, was analyzed with RSTRIP program (MicroMath Scientific Software, Salt Lake City, Utah) using a single compartment model and zero weighting. The decay half-lives are given in Table 4.

25 A comparison of the data shown in Table 4 with the 82 mM (initial cytarabine concentration) formulation and the 246 mM (initial cytarabine concentration) shows that the total amount of cytarabine decreases more rapidly for the 82 mM formulation than for the 246 mM formulation. The concentration in the pellet fraction also decreases more rapidly for the 82 mM formulation than for the 246 mM

formulation. Therefore, this study shows that the rate of release of cytarabine decreases with increasing osmolarity of the first aqueous component, not only in the physiologically relevant environment, namely, human plasma as shown in EXAMPLE 1, but also *in vivo*.

5

TABLE 4

Concentration of cytarabine in the pellet fractions of the peritoneal fluid, and the total amount of cytarabine in the peritoneal cavity, at different times subsequent to intraperitoneal injection of formulations prepared using a solution containing either 82 mM cytarabine or 246 mM cytarabine. A group size of three was used.

10

Hours After Intraperitoneal Injection	Concentration in Pellet Fraction ($\mu\text{g/mL}$)		Total Amount (μg)	
	82 mM	246 mM	82 mM	246 mM
	Formulation	Formulation	Formulation	Formulation
0	9,380	9,685	2,372	2,791
15	0.5	9,925 \pm 371	6,896 \pm 1,083	1,108 \pm 67
	4	1,115 \pm 1,108	13,360 \pm 761	150 \pm 14
	15	1,015 \pm 922	11,493	76 \pm 58
	64	ND	1,493 \pm 132	ND
	108	ND	634 \pm 518	ND
20	$t^{1/2}$ (r^2)	1.7 (0.974)	29.6 (0.904)	0.64 (0.989)
				13.6 (0.892)

ND, not determined; $t^{1/2}$, Decay Half-Life in Hours.

25

While presently preferred embodiments of the invention have been given for the purpose of disclosure, changes may be made therein which are within the spirit of the invention as defined by the scope of the appended claims.

CLAIMS

1. A process for producing multivesicular liposomes comprising the steps of:
 - (a) forming a water-in-oil emulsion from two immiscible components, a lipid component comprising at least one organic solvent, at least one amphipathic lipid and a neutral lipid, and a first aqueous component comprising at least one biologically active substance;
 - (b) dispersing the water-in-oil emulsion into a second aqueous component to form solvent spherules;
 - (c) removing the organic solvent from the solvent spherules to form the multivesicular liposomes;

wherein the osmolarity of the first aqueous component is chosen to modulate the rate of release from multivesicular liposomes into a physiologically relevant aqueous environment.

2. The process of claim 1 wherein, the amphipathic lipid is an amphipathic lipid with a net negative charge.
3. The process of claim 2 wherein
the amphipathic lipid is selected from the group consisting of cardiolipins, phosphatidylserines, phosphatidylglycerols, phosphatidylinositols and phosphatidic acids.
4. The process according to claim 2 wherein,
the lipid component further comprises a sterol.

5. The process according to claim 4 wherein,
the lipid component further comprises a zwitterionic lipid.
6. The process according to claim 1 wherein,
the amphipathic lipid is a zwitterionic lipid.
7. The process according to claim 5, wherein
the zwitterionic lipid is selected from the group consisting
of phosphatidylcholines, phosphatidylethanolamines,
sphingomyclins, and lysophosphatidylcholines.
8. The process according to claim 1 wherein, the amphipathic lipid
has a net positive charge.
9. The process according to claim 8, wherein
the amphipathic lipid is selected from the group consisting
of diacyl trimethylammonium propanes, diacyl
dimethylammonium propanes and stearylamine.
10. The process of claim 1, 2, 4, 6 or 8 wherein,
the first aqueous component further comprises an osmotic
spacer to increase the osmolarity of the first aqueous
component.
11. The process according to claim 1, 2, 4, 6 or 8 wherein,
the lipid component is selected from the group consisting
of a phospholipid and an admixture of phospholipids.
12. The process of claim 1 or 6 wherein, the physiologically relevant
aqueous environment is a storage medium.

- 5
13. The process of claim 1 or 6 wherein,
the physiologically relevant aqueous environment is selected from the group including but not limited to normal saline, buffered saline, human plasma, serum, cerebrospinal fluid, subcutaneous fluid, synovial fluid, intraocular fluid, intraperitoneal fluid, intramuscular fluid, or combinations thereof.
 14. The process according to claim 1 wherein,
at least one of the amphipathic lipids has a net negative charge.
 15. The process according to claim 1 wherein,
the amphipathic lipid is provided in admixture with cholesterol.
 16. The process according to claim 1 wherein,
a lipophilic biologically active material is provided in admixture with the lipid component.
 17. The process according to claim 11 wherein,
the neutral lipid is selected from the group consisting of triolein, tricaprylin, soybean oil, lard, beef fat, tocopherol, squalene, and combinations thereof.
 18. The process according to claim 1 wherein,
the organic solvent is selected from the group consisting of ethers, hydrocarbons, halogenated hydrocarbons, halogenated ethers, esters, CO₂, NH₃, Freons, and combinations thereof.

19. The process according to claim 1 wherein,
the biologically active material is hydrophilic.
20. The process according to claim 1 wherein,
the emulsification of the two said components is carried
out using a method selected from the group consisting of
mechanical agitation, ultrasonic energy, and nozzle
atomization.
21. The process according to claim 20 wherein,
the average size and number of the aqueous chambers
within the vesicles are determined by time and duration of
the method of emulsification selected.
22. The process according to claim 1 wherein,
the first aqueous component consists essentially of water
and the biologically active substance.
23. The process according to claim 1 wherein,
the second aqueous component comprises substances
selected from the group consisting of free-base lysine,
free-base glycine, and free-base histidine, and a
combination thereof; and

substances selected from the group consisting of glucose,
sucrose, trehalose, succinate, cyclodextrin, arginine,
galactose, mannose, maltose, mannitol, glycine, lysine,
citrate, sorbitol, dextran, sodium chloride, and
combinations thereof.

24. The process according to claim 1 wherein,
the second aqueous component comprises substances
selected from the group consisting of monosaccharides,
disaccharides, polysaccharides, and other polymers.
25. The process according to claim 1 wherein,
the removing of the organic solvent is by a solvent
removal system selected from the group consisting of
sparging, evaporation, passing gas over the solvent
spherules, spray drying, and combinations thereof.
26. The process of claim 1 or 6 wherein,
the biologically active substance is selected from the
therapeutic categories consisting of antianginas,
antibiotics, antihistamines, antineoplastics, monoclonal
antibodies, radionuclides, tranquilizers, antiarrhythmics,
antidiabetics, antihypertensives, antivirals, hormones,
neurotransmitters, antiasthmas, antifungals, antiparasitics,
cardiac glycosides, immunomodulators, nucleic acids and
analogues, radio contrast agents, steroids, vasopressors,
vaccines, sedatives and analgesics, and combinations
thereof.
27. The process of claim 1 or 6 wherein,
the biologically active substance is cytarabine.
28. The process of claim 1 or 6 wherein,
the biologically active substance is amikacin.
29. The process of claim 1 or 6 wherein,
the biologically active substance is selected from the
group consisting of therapeutic proteins and peptides.

-31-

30. The process of claim 1 or 6 wherein,
the biologically active substance is selected from the
group consisting of herbicides, pesticides, fungicides,
insecticides, and combinations thereof.
31. The process of claim 1 or 6 wherein,
the biologically active substance is selected from the
group consisting of nucleic acids.
32. The process of claim 1 or 6 wherein,
the biologically active substance is selected from the
group consisting of cosmetics, perfumes, moisturizers,
and combinations thereof.
33. The multivesicular liposome of claim 1 or 6 having an attached
target specific ligand, or hydrophilic coating.
34. A method for the treatment of a patient with a biologically active
compound comprising,
administering to the patient the said compound
encapsulated in a multivesicular liposome of claim 1 or 2.
35. A method for the treatment of a patient with a biologically active
compound comprising,
administering to the patient a multivesicular liposome of
claim 25, 26, 29 or 30.
36. A multivesicular liposome having a first aqueous component
comprising a biologically active substance encapsulated therein
for sustained release wherein the multivesicular liposome is in a
physiologically relevant aqueous environment and wherein the
osmolarity of the first aqueous component is increased to

decrease the rate of release of the active substance into the physiologically relevant aqueous environment, or wherein the osmolarity of the first aqueous component is decreased to increase the said rate of release.

37. A multivesicular liposome of claim 34 wherein,
the first aqueous component comprises sufficient
biologically active substance to attain the desired
osmolarity.
38. A multivesicular liposome of claim 35 wherein,
the first aqueous component further comprises sufficient
osmotic spacer to attain the desired osmolarity.
39. A method for controlling the rate of release of an active agent
from a multivesicular liposome into a physiologically relevant
aqueous environment comprising adjusting the osmolarity of the
first aqueous component so as to decrease the rate of release
of the active agent from the liposome, with increasing osmolarity
providing a decreasing release rate.
40. The method of claim 39 wherein,
an osmotic spacer is introduced into the first aqueous
component to augment the osmolarity thereof.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/11609

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 9/127

US CL : 424/450; 264/4.1, 4.3

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/450; 264/4.1, 4.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONEElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)
NONE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, 4,224,179 (SCHNEIDER) 23 September 1980, see abstract, columns 7 and 8 and Example I.	1-22, 25-26, 29, and 34-35
X	US, A, 5,094,854 (OGAWA ET AL.) 10 March 1992, see abstract, columns 3 and 4 and claims.	36-40
--		-----
Y		1-35
Y	Arch Ophthalmol, Volume 105, issued March 1987, ASSIL ET AL., "MULTIVESICULAR LIPOSOMES", pages 400-403, see column 2 on page 400.	1-40

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	
A document defining the general state of the art which is not considered to be of particular relevance	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
E earlier document published on or after the international filing date	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
O document referring to an oral disclosure, use, exhibition or other means	*Z* document member of the same patent family
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

07 NOVEMBER 1995

Date of mailing of the international search report

07 DEC 1995

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

G. S. KISHORE

Telephone No. (703) 308-1351